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RECENT ADVANCES IN THE KNOWLEDGE OF ANTI-ANEMIA FACTORS

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RECENT ADVANCES IN THE KNOWLEDGE OF ANTI-ANEMIA FACTORS

By RUTH WOODS

Pernicious anemia has been recognized for one hundred years. The role of diet in its treatment and cure has been known for twenty years. In the last five years of this century-long investigated disease, research discoveries have been catapulted forth with ever-increasing momentum. Beginning with studies which culminated in the synthesis of folic acid* in 1945, the first of the final chapters in the story of the anemias was written. In swift succession came studies outlining the anti-anemia role of related compounds such as xanthopterin, pyracin and thymine as well as several derivatives of folic acid itself. Barely three years later in 1948 came a breath-taking climax with the discovery of Vitamin B_{12}**, hailed at last as *the* anti-pernicious anemia factor. In January of this year, coming almost as an anti-climax, announcement was made of yet another new factor, vitamin B_{14}.

A little more than twenty years ago, victims of pernicious anemia died not knowing that their lives might have been saved by a common food, liver, then often considered an article of diet fit only for household pets. Today even the layman knows that anemias may be treated with liver extract, folic acid, and vitamin B_{12}. With the advent of vitamin B_{14} into the picture of anti-anemia factors, even the trained scientist must pause to assess a conglomeration of findings which even now do not yet constitute a precise picture of the anti-anemia story. Many questions present themselves: Why is folic acid limited in its curative abilities? What is the relation between folic acid and Vitamin B_{12}? What part do the compounds thymine and thymidine play in this complex story? Can the classic "intrinsic" and "extrinsic" factors and the so-called "anti-pernicious anemia principle" of liver be identified with folic acid, B_{12}, their derivatives or products of their chemical interactions? Is vitamin B_{12} really the anti-pernicious anemia factor and, if so, where does vitamin B_{14} fit into the picture? How do any or all of these elements operate independently, in relation to each other, and most of all, in the cure

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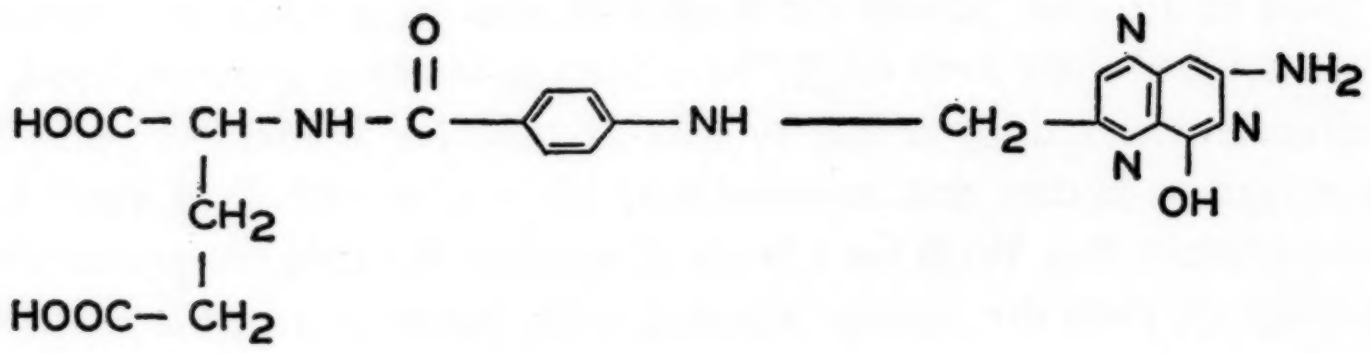
**cf. Borden's Review of Nutrition Research,* Feb., 1946, "Folic Acid."

***cf. Borden's Review of Nutrition Research,* Jan. 1949, "The Story of Vitamin B_{12}." On November 7, 1949 announcement was made of the chemical nature of one fraction of the B_{12} molecule by Folkers (1). This fraction has been identified as 5, 6-dimethylbenzimidazole. It is of interest to note that the parent component of this compound (1, 2-diamino-5, 6-dimethylbenzene) is also part of the molecular structure of riboflavin.

of anemia? The present Review will attempt to assess the present status of the known and postulated anti-anemia factors and, at the same time, to provide an answer to some of the puzzling questions just enumerated.

Folic Acid: The experimental investigations leading to the discovery of folic acid and the clinical studies outlining its value in the treatment of human anemias have been reviewed in detail in earlier issues of this publication* and will not be repeated here. Almost simultaneously with the publication of these reviews came the chemical identification of folic acid (specifically the *L. casei* factor from liver) as a compound having a basic pteridine nucleus to which was linked p-amimobenzoic acid and a molecule of glutamic acid (1, 2, 3, 4). On the basis of this chemical structure folic acid was named *pteroylglutamic acid.* Other members of the folic acid complex were found to be derivatives of pteroylglutamic acid differing only in the number of glutamic acid molecules. The basic structure for these compounds was named *pteroic acid.* (Fig. 1). Recently the name folic acid or folacin has been officially adopted to designate the *L. casei* factor (pteroylglutamic acid).

Fig. 1 **Folic Acid (FOLACIN)**



N-[4-{[(2-AMINO-4-HYDROXY-6-PTERIDYL) METHYL] AMINO} BENZOYL] GLUTAMIC ACID

LIVER L. CASEI FACTOR

PTEROYLGLUTAMIC ACID

The "folic acid" group consists of four major compounds:

1. Pteroylglutamic acid { natural *L. casei* factor from liver; possesses one molecule of glutamic acid.

*cf. *Borden's Review of Nutrition Research,* Feb. and April, 1946, "Folic Acid" and "The Clinical Use of Folic Acid."

2. Pteroyltriglutamic acid ("teropterin") — fermentation *L. casei* factor obtained from a microorganism; has three molecules of glutamic acid.

3. Pteroylheptaglutamic acid — vitamin B_c conjugate from yeast; has six *extra* glutamic acid molecules. This form can be utilized only by those species possessing an enzyme (vitamin B_c conjugase) which splits off the six extra molecules, leaving pteroylglutamic acid.

4. Pteroic acid — a basic synthetic form obtained by substituting p-aminobenzoic for p-aminobenzoylglutamic acid in the synthesis.

Xanthopterin: Originally discovered as a pigment in yellow butterfly wings, and later in many other biological materials, the pteridine compound known as *xanthopterin* has long held a place of interest among biochemists. Because of its widespread occurrence, much speculation was aroused as to a possible physiological role for this compound. The search for such a role for xanthopterin was given great impetus with the discovery that the basic nucleus of this compound and that of folic acid were identical, namely a pteridine base.

Xanthopterin and Anemia: Even before the knowledge of this coincidence, however, a number of studies had pointed to a role of xanthopterin in the cure of certain types of experimental anemia. As early as 1936, Tschesche and Wolfe (5, 6) abroad, reported that xanthopterin cured an anemia in rats produced by a diet of goat's milk. Some five years later, Norris and Simmons (7, 8) in the United States found that injections of xanthopterin caused a rapid rise in red blood cells in anemic fish, with subsequent return to normal levels.

Although Totter and Day (9) reported further in 1943 that xanthopterin alleviates the leukopenia (decrease in white blood cells) resulting from the feeding of sulfathiazole to rats, this was not confirmed by others (10, 11). The latter two groups of investigators (10, 11) did find, however, that this type of leukopenia, responded to folic acid. Thus, even before knowledge of their close chemical relationship was uncovered, biological studies hinted at a possible tie-up between folic acid and xanthopterin in the treatment of anemias.

Folic Acid—Xanthopterin Interrelations:

Following the chemical identity of folic acid, the activities of this vitamin and the related pteridine compound, xanthopterin, were studied in some

detail by Norris and Majnarich. Their first studies were made with cultures of bone marrow and were designed to compare the effects of xanthopterin, folic acid and normal blood serum on the stimulation of new cell growth in bone marrow (12). Three types of bone marrow cells were studied—red blood cells, white blood cells and reticulocytes.* Cell counts were made after varying periods (zero, three and six hours) of supplementation of the bone marrow cultures. Striking increases in cell counts of all three types were observed in those cultures to which xanthopterin or blood serum had been added. Results with folic acid showed no appreciable increase over those observed in unsupplemented control cultures. (Table 1)

TABLE 1

Initial and Final Red Blood Cell Counts in Bone Marrow Cultures

	Initial	Final
Unsupplemented Controls	21×10^4	23×10^4 cells per c. ml.
Folic acid supplemented —(5 mcgm./ml.)	21×10^4	24×10^4 cells per c. ml.
Xanthopterin —(5 mcgm./ml.)	21×10^4	32×10^4 cells per c. ml.
Blood serum —(0.1 ml./ml.)	21×10^4	37×10^4 cells per c. ml.

The pronounced effect of xanthopterin on cell proliferation led to a further study on the value of this material in sulfathiazole-induced anemia in rats (13). As was expected, excellent increases were noted in red cell count, hemoglobin and hematocrit readings. Comparison of the effects of injected xanthopterin and pteroylglutamic acid, indicated an immediate response to the former, and a delayed response (following a 3 to 5-day lag) of equal magnitude to folic acid.

From the observation that folic acid was ineffective in stimulating cell proliferation in bone marrow (12) whereas xanthopterin was immediately active, the suggestion was made that folic acid must first be degraded to xanthopterin before it can be used by the bone marrow to form new blood cells.

Optimum Dosage Levels: These comparative effects of xanthopterin and folic acid were again demonstrated in quantitative studies with anemic rats designed to find the optimum dosage level for xanthopterin. This level was found to be about 1 mg. of xanthopterin per kilo of body weight (14). Doses up to 5 mg./k. promoted red cell growth in anemic rats. Best results,

*The reticulocyte is believed to be a structural variation of the normal red blood cell and is thought to represent a stage in the maturation of the red blood cell. For this reason, an increase in reticulocytes is usually accepted as a sign of red cell formation even before any increase in red cells or hemoglobin is noted.

however, were obtained at the 1 mg. level. Doses over 10 mg./k. aggravated the anemia. When 1 mg. to 5 mg./k. doses of xanthopterin were given to *normal* rats, polycythemia (excess red blood cells) and leukocytosis (excess white cells) occurred. Doses over 5 mg./k. produced anemia and leukopenia in normal rats. Again, folic acid was found, under similar conditions, to be less active in curing anemia in the rat than xanthopterin.

The failure of some investigators to observe a favorable effect of xanthopterin in animals and the similar lack of response to xanthopterin in human clinical studies (15)* may conceivably be attributed to administration of dosages outside the narrow optimum range in which Norris and Majnarich have demonstrated xanthopterin to be effective.

At present, the metabolic interrelationships between folic acid and xanthopterin remain still unsettled. Both compounds occur naturally and have closely similar structures. The rather clear-cut studies of Norris and Majnarich which seem to indicate that xanthopterin is a possible "active" form of the precursor, folic acid, must await confirmation by other investigators with both animal and human subjects before this question can be conclusively resolved.

Another possible mechanism which should not be overlooked has been suggested by the studies of Wright, Skeggs and Welch (17). They noted that the folic acid content of rat liver tissue is markedly increased by the presence of xanthopterin. Their observations led to the theory that the free folic acid in rat liver is converted to an inactive form by liver enzymes. This reaction (which did not occur in rat muscle tissue) was found to be inhibited by several factors, one being the presence of xanthopterin. The authors point out that while these studies do not exclude the possibility of folic acid formation from xanthopterin, the data suggest a metabolite-antimetabolite relationship or mass inhibition of enzymatic activity.

Folic Acid and Thymine:

Paralleling the studies on the folic acid-xanthopterin relationship, were microbiological experiments which revealed a new facet of the biochemical behavior of the new vitamin. This was the revelation of an important interrelationship between folic acid and the pyrimidine base, thymine. This relationship was first uncovered in nutrition studies with lactic acid bacteria. In 1941, Snell and Mitchell (18) reported that thymine was essential in the culture medium for *S. lactis*. This constituted the first time that thymine was

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*Although in this study (15) no response of anemia in human subjects could be observed, Norris and Majnarich (16) subsequently confirmed their studies on cell proliferation stimulation in animal bone marrow by similar findings in *in vitro* tests with human bone marrow.

recognized as a growth essential for any living organism. At about the same time, it was shown that *S. lactis, L. casei* and other lactic acid bacteria require folic acid for growth (19). It soon became apparent, however, that under certain conditions, thymine (or its riboside derivative, thymidine) could successfully replace folic acid as a growth essential in the nutrition of these microorganisms (20). At least 5,000 times more thymine than folic acid was required.

In connection with these findings several unusual observations were noted. The absence of folic acid in the thymine-grown microoganisms was the first recorded exception to the occurrence of this growth factor in living cells. Further, the full replaceability of folic acid by thymine without conversion of the latter into folic acid is also unusual. This led to speculation as to the biological role of folic acid in these microorganisms. As Stokes concluded:

> "Folic acid is probably converted into a coenzyme or functions directly as one since as little as 0.002 microgram unit per ml. of medium is sufficient for half-maximum growth. Thymine, however, is required in amounts at least 5,000 times as much . . . Three to four mcgm. of thymine were taken up . . . during the synthesis of each milligram of *S. lactis* cells and the assimilated thymine could not be extracted . . . This thymine content compares favorably with the 2 mcgm. level found as nucleic acid per mg. of dried tubercle bacilli. The quantity of thymine required . . . is therefore sufficiently large to serve directly as a building block for the synthesis of the bacterial nucleic acid. Folic acid would, according to this idea, function as a coenzyme for the enzyme system responsible for the synthesis of thymine . . . which, in turn, is used . . . to form nucleic acid. This would explain why in the presence of thymine, folic acid is no longer necessary for growth; the synthesis of thymine becomes superfluous in the presence of an extraneous supply of the substance."

In other words, providing large amounts of thymine would make possible the direct utilization of this material as a building block for nucleic acid without the presence of folic acid as a catalyst for this synthesis.

Thymine and the Treatment of Anemia: Stokes' hypothesis concerning the replaceability of folic acid by large amounts of thymine in the basic biological processes of microorganisms suggested its use in proportionately large quantities as a substitute for folic acid in human metabolism. In 1946, Spies and coworkers (21) reported the successful response of a single case of nutritional macrocytic anemia to large doses of thymine (6 grams per day for several weeks). The dose was only about one-fourth to one-half the calculated dosage (on a 5,000: 1 ratio) because of the scarcity of thymine, nevertheless peak reticulocyte responses were obtained after only 11 days

and maximum red blood cell and hemoglobin responses occurred on the twentieth day.

These microbiological and clinical findings, limited though they are, suggest a possible mechanism explaining the action of both thymine and folic acid in the stimulation of blood cell formation through the synthesis of nucleic acid, a fundamental component of all living cells.

In view of the very recent studies indicating a possible conversion of folic acid to xanthopterin before the former can be utilized (12, 13, 14, 16) it is interesting to speculate on the possible enzymatic role of xanthopterin in thymine synthesis and to wonder whether xanthopterin might replace thymine in the same manner as, and, perhaps in lower concentration than, folic acid.

Vitamin B_{12}, Folic Acid and Thymidine:

During studies by Shive *et al* (22) on factors functionally related to folic acid, a crystalline factor was isolated from liver which was found to prevent the toxic action of methyl-folic acid (a folic acid antagonist). The new factor was found to be several times as active as folic acid in stimulating the growth of a test microorganism. Chemical investigation revealed the factor to be *thymidine* (thymine desoxyriboside). Although discovered as a result of independent studies, this finding confirmed earlier studies indicating the replaceability of folic acid by both thymine and its riboside, thymidine.

Soon afterward, announcement of the isolation of vitamin B_{12} (the anti-pernicious anemia factor) as a microbial growth factor (23, 24) led Shive *et al* (25) to a study of the vitamin to determine whether or not it was identical with a factor found in their laboratory which was involved in the biosynthesis of thymidine. Their experiments revealed that vitamin B_{12} could be replaced by thymidine in the nutrition of the test microorganism.

They concluded, therefore, that vitamin B_{12} probably functions in the biosynthesis of thymidine.

If this assumption is correct, then vitamin B_{12} plays a role in the synthesis of thymidine similar to the one played by folic acid in the synthesis of thymine. One interesting point may be noted: Although folic acid may be replaced by either thymine or thymidine, vitamin B_{12} is replaceable only by thymidine.*

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*In a paper published a short time later, Shive *et al* (25) indicate that *L. lactis Dorner* requires, in the absence of vitamin B_{12}, not only thymidine (a pyrimidine) but also certain purines, notably guanylic acid or mixtures of (1) adenine and guanine or (2) hypoxanthine and guanine. B_{12} completely replaced the need for both purines and pyrimidine, although the purines had a slight sparing action on the need for B_{12}. This was interpreted to mean that B_{12} is concerned not only with pyrimidine (thymidine) synthesis but with purine synthesis as well. The

Confirmation of the effectiveness of thymidine in replacing B_{12} in the nutrition of certain lactobacilli and of the probable role of B_{12} in its biosynthesis was obtained through the work of Wright, Skeggs and Huff (26). They postulated further that:

> "The primary biochemical defect in pernicious anemia may well be the inability to synthesize certain nucleosides, particularly thymidine, from parent purines and pyrimidines."
>
> "Thus it would appear that the curative effects observed in this disease with folic acid arise from increased thymine synthesis which, by mass action effects, yields more thymidine. The effects of large amounts of thymine in pernicious anemia similarly may be explained."

Relation of These Factors to Pernicious Anemia: Thus folic acid is brought into direct interplay with vitamin B_{12} in the anemia story. The key compound in the story is, apparently, thymidine, which is needed for the manufacture of nucleic acids for new cells (i.e. such as new red blood cells, reticulocytes, etc.). Folic acid makes possible increased thymine synthesis. Increased thymine makes possible an increase in the synthesis of thymidine. This latter synthesis is further aided by the catalytic action of vitamin B_{12}. With the greatly increased synthesis of thymidine, basic material is available for making nucleic acids to be used in the manufacture of new cells to overcome the shortage characteristic of anemia.

Vitamin B_{14}, and other Hematopoietic Factors:

Announcement of a new hematopoietic substance, vitamin B_{14}, was made in January of this year, by Norris and Majnarich (28). Although by no means resolving the complex picture of anti-anemia factors and the mechanisms whereby they operate, this work (28, 29) serves to tie in several loose ends pertaining to the interrelationships among folic acid, xanthopterin and other factors and also suggests some possible interpretations in relation to the classic "intrinsic" and "extrinsic" factors in pernicious anemia.

Intrinsic-Extrinsic Factors: According to the classic theories of Castle and associates (30, 31) two factors are important in pernicious anemia.*

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possibility of a reverse action—i.e. the role of purines and pyrimidines in B_{12} synthesis,—however, was not precluded.

A further interesting finding was reported as a result of studies with the microorganism, L. leichmannii (used in early studies leading to isolation of the animal protein factor—*cf. Borden's Review of Nutrition Research,* Oct., 1948, "The Animal Protein Factor"). Thymidine may replace the animal protein factor in this organism. Shive *et al* (27) found, further, that folic acid was needed in a medium containing purines. This need for folic acid could be replaced, after a lag period, by thymine. The need for the animal protein factor (vitamin B_{12}) was replaced by thymidine in the presence of folic acid, or very slowly, by thymidine alone.

**cf. Borden's Review of Nutrition Research,* Mar., 1946, "Nutritional Milestones in the Anemias."

The intrinsic factor, a substance in normal gastric juice, and the extrinsic factor, a substance provided by certain foods. Thus, patients suffering from a deficiency of gastric juice, (and hence, lack of intrinsic factor) fail to utilize the extrinsic factor of foods and develop anemia. Until recently, it was believed that the operative mechanism involved the production of an "anti-anemia principle" formed by the interaction of the intrinsic and extrinsic factors (32). The anti-pernicious anemia principle was believed to occur preformed in liver. Since the potency of both crude and refined liver extracts could be further increased by incubation with gastric juice, it was believed that these contain the extrinsic factor as well as the "preformed" liver factor.

More recently with the isolation of pure vitamin B_{12}, believed to be identical with the "preformed" anti-pernicious anemia factor, Berk, Castle *et al* (33) were able to demonstrate clinically a similar increase in activity of the pure vitamin when administered with normal human gastric juice. *They suggest therefore that the food (extrinsic) factor may be identical with the anti-pernicious anemia principle of liver which, itself, is presumably identical with vitamin* B_{12}. Further, it is suggested that the function of the intrinsic factor is *to increase absorption of vitamin* B_{12} *rather than to react with it to form an anti-anemia substance as hitherto assumed.* In other words, there may be no "preformed liver factor" at all, but merely the extrinsic factor (vitamin B_{12}) and the intrinsic factor (in gastric juice) necessary for the complete absorption of B_{12}. Both Berk *et al* (33) and Bethell *et al* (34) report large fecal losses of B_{12} in patients with untreated pernicious anemia. This would seem to confirm the hypothesis that normal gastric juice aids the absorption of the vitamin or protects it from destruction in the upper gastrointestinal tract.

Vitamin B_{14}: This new factor was isolated in crystalline form from urine. It was found to be extremely active in stimulating the proliferation of new cells in bone marrow cultures, being of the order of five million times as potent as xanthopterin. B_{14} was found also to be effective in curing experimentally induced anemia of rats. Both B_{14} and xanthopterin were inhibited by the anti-folic acid compound, 7-methylfolic acid, thereby indicating a close relationship between the two former substances.

Further studies revealed that enzymes from milk, liver and gastric mucosa act on xanthopterin, folic acid and teropterin (pteroyltriglutamic acid) to produce possibly vitamin B_{14}, or at least related substances which exhibit activity identical with that of B_{14} (29).

From these observations, Norris and Majnarich deduce that the extrinsic factor may be one of a related group of compounds, namely: xanthopterin,

folic acid, teropterin or other pteroyl derivates and that the intrinsic factor may be one of several enzymes such as xanthine oxidase from milk or certain similar enzymes in liver and stomach tissue. They believe that the extrinsic and intrinsic factors react to form an active anti-anemia substance which may be vitamin B_{14} or a closely related compound. This theory supports the classic concept of Castle who, however, in recent work, (33) has replaced this theory with the assumption that the extrinsic factor (vitamin B_{12}) is the active anti-anemia substance and that the function of the intrinsic factor is not to combine with B_{12} to form a new substance but rather to facilitate its absorption in the body. Norris and Majnarich do not discuss the position of B_{12} in the anemia story or relate it to their proposed scheme of biochemical reactions.

Conclusion:

With the discovery of folic acid, vitamin B_{12}, and related anti-anemia material, the final conquest of pernicious anemia through nutrition research has been accomplished. The discovery of these compounds and their mode of action has also shed considerable light on the probable mechanisms involved in the production of this disease. It is quite likely, therefore, that research with these new nutrition tools will completely clarify the century-old mystery of the biochemical nature of pernicious anemia, its causes and cure, in the very near future.

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